† Electronic Supplementary Information (ESI)

Neutral Merocyanine Dyes: for In Vivo NIR Fluorescence Imaging of Amyloid-β Plaques

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Contents

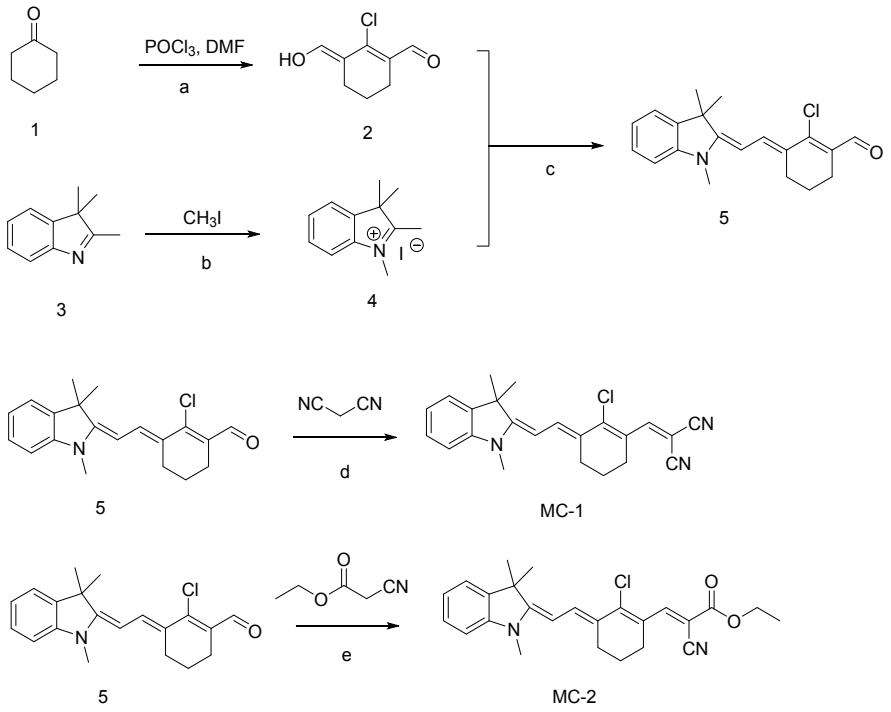
General Information

All solvents and reagents (analytical grade) were obtained commercially without further purification unless otherwise mentioned. Column-layer chromatographic silica gel was purchased from Branch of Qingdao Haiyang Chemical Co., Ltd. ^{1}H and ^{13}C NMR spectra were recorded using TMS as the internal standard in $CDCl_3$ or DMSO-d_6 with a Bruker BioSpin GmbH spectrometer at 400 MHz and 100 MHz, respectively. High resolution mass spectra (HRMS) were taken on a Thermo-Fisher LTQ Orbitrap XL instrument. Flash column chromatography was performed with silica gel (200-300 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd. All chemicals were purchased from commercial sources unless otherwise specified.

The synthetic trifluoroacetic acid salt forms of $A\beta_{(1–42)}$ peptides, human serum albumin (HSA) and bovine serum albumin (BSA) were purchased from Qiang Yao Biological Technology Co. (China). Acetylcholinesterase (AChE, E.C. 3.1.1.7, from electric eel), butyrylcholinesterase (BuChE, E.C. 3.1.1.8, from equine serum) were purchased from Sigma–Aldrich. $A\beta_{(1-42)}$ powder was dissolved in 0.1% ammonium solution and stored at -80 ºC until used. The aggregated $A\beta_{(1-42)}$ was prepared by diluting the stock solution of $A\beta_{(1-42)}$ to 200 μM with PBS solution (pH 7.4) and incubating at 37 ºC for 7 days. The composition of artificial cerebrospinal fluid (aCSF) was NaCl (124 mM), KCl (3 mM), NaH_2PO_4 (1.25 mM), $MgCl_2$ (1 mM), $NaHCO_3$ (36 mM), D-glucose (10 mM), $CaCl_2$ (2 mM), and bubbled with 95% O_2/5% CO_2.

The UV-vis absorption spectra were recorded on a UV-2450 spectrophotometer (Shimadzu, Japan). Fluorescence measurements were performed on an FL-4500 fluorescence spectrophotometer (Hitachi, Japan) and Fluoro Max-4 (HORIBA, Japan) equipped with quartz cell of 10.0 mm path length. Fluorescence QYs were measured using an ethanol solution of Rhodamine B as a standard.

Synthesis and characterization of probes



Scheme S1. The synthetic route of **MC-1** and **MC-2**

Reagents and conditions: (a) DMF, 80℃ reflux. (b) EtOH, 130℃. (c) Toluene: Butyl alchohol=2.5:1, 130℃, reflux. (d) Saturated K_2CO_3 in MeOH, MeOH, rt. (e) Piperidine, MeOH, 60℃.

Synthesis of Compound 2. Under N_2 atmosphere $POCl_3$ (15 mL, 0.058 mol) was slowly added in the dichloromethane solution (18 mL) containing *N*, *N*-dimethylformamide (18 mL, 0.26 mol) in an ice bath. After stirring for 30 min, cyclohexanone (4.5 mL, 0.048 mol) was added, and the ice bath and N_2 atmosphere were removed. The resulting solution was stirred at 80℃ for 4h. The solution was cooled down to room temperature, followed by the addition of icy water. Light yellow solid **2** was obtained (7.5 g, 90.8%). ^{1}H NMR (400 MHz, DMSO) δ 2.51 (s, 1H), 2.39 - 2.28 (m, 4H), 1.62-1.52 (m, 2H).

Synthesis of Compound 4. An ethanol solution (60 mL) of 2,3,3-trimethyl- indolenine (4.8 mL, 0.03 mol) and iodomethane (3.3 mL, 0.05 mol) was added into a pressure vessel. The sealed vessel was heated to 80℃ overnight. Cooling to room temperature, a violet crystal **4** was formed and the crystal was filtered (7.7 g, 85.3%). ^{1}H NMR (400 MHz, DMSO) δ 7.92-7.86 (m, 1H), 7.85-7.78 (m, 1H), 7.66-7.58 (m, 2H), 3.96 (s, 3H),

2.76 (s, 3H), 1.52 (s, 6H).

Synthesis of Compound 5. Compounds **2** (7.0 g, 0.04 mol) and **4** (7.5 g, 0.025 mol) were dissolved in a mixture of toluene and 1-butanol (v/v = 1:2.5). The resulting solution was refluxed at 130℃ in the sealed pressure vessel. The reaction was monitored by TLC plate. After cooling to room temperature, the solution was concentrated under reduced pressure. The crude product was purified by column chromatography to obtain compound **5** (2.0 g, 24.5%) as a red solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.28 (s, 1H), 7.85 (d, *J* = 12.5 Hz, 1H), 7.23 (t, *J* = 3.5 Hz，1H), 7.22 (d, *J* = 3.7 Hz, 1H), 6.96 (t, *J* = 7.3 Hz, 1H), 6.75 (d, *J* = 7.7 Hz, 1H), 5.48 (d, *J* = 12.6 Hz, 1H), 3.25 (s, 3H), 2.61 (t, *J* = 5.3 Hz, 2H), 2.51 (t, *J* = 5.6 Hz, 2H), 1.83-1.76 (m, 2H), 1.68 (s, 6H).

Synthesis of Compound MC-1. To a methanol solution containing compound **5** (50.2 mg, 0.15 mmol) and malononitrile (99 mg, 1.5 mmol), saturated K_2CO_3 methanol solution (40 μL) was added. The solution was stirred at room temperature (monitored by TLC plate) until the starting materials disappeared. A blue crystal was obtained and was filtered to give **MC-1**. (80 mg, 71.1%). ^{1}H NMR (400 MHz, DMSO) δ 8.00 (d, J = 13.8 Hz, 1H), 7.82 (s, 1H), 7.48 (d, J = 6.9 Hz, 1H), 7.33 (t, J = 7.7 Hz, 1H), 7.20 (d, J = 7.6 Hz, 1H), 7.10 (t, J = 7.4 Hz, 1H), 5.90 (d, J = 12.9 Hz, 1H), 3.47 (s, 3H), 2.81 (t, J = 8.7 Hz, 2H), 2.60 (t, J = 9.3 Hz, 2H), 1.85-1.73 (m, 2H), 1.60 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.28, 153.40, 148.14, 143.74, 139.64, 137.59, 128.17, 124.81, 123.96, 122.35, 121.94, 117.37, 115.59, 107.87, 95.15, 72.11, 47.32, 29.82, 29.71, 28.34, 27.49, 25.86, 20.96. Purity: 99.56% by HPLC. HRMS (ESI): calcd for $(M+H)^+$ ($C_{23}H_{23}N_3Cl^+$) 376.1575, found 376.1585.

Synthesis of Compound MC-2. To a methanol solution containing compound **5** (82.3 mg, 0.25 mmol) and ethyl cyanoacetate (0.283 ml, 2.5 mmol) piperidine (100 μL, 1.1 mmol) was added. The solution was refluxed at 60℃ (monitored by TLC plate) until the starting materials disappeared. After cooling to room temperature, the solution was concentrated under reduced pressure. The crude product was purified by column

chromatography to obtain **MC-2** (50 mg, 47.4%). ^{1}H NMR (400 MHz, DMSO) δ 8.45 (s, 1H), 7.86 (d, J = 14.1 Hz, 1H), 7.42 (d, J = 7.6 Hz, 1H), 7.28 (t, J = 8.2 Hz, 1H), 7.09 (d, J = 8.7 Hz, 1H), 7.02 (t, J = 7.1 Hz, 1H), 5.75 (d, J = 12.9 Hz, 1H), 4.25 (q, J = 14.5, 7.5 Hz, 2H), 3.37 (s, 3H), 2.88 (t, J = 5.8 Hz, 2H), 2.60 (t, J = 8.3 Hz, 2H), 1.79 (m, 2H), 1.59 (s, 6H), 1.26 (t, J = 7.5 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.13, 164.59, 164.31, 164.12, 151.40, 147.50, 147.22, 144.13, 139.45, 134.67, 134.38, 128.00, 125.24, 124.41, 121.85, 121.55, 107.26, 94.28, 61.93, 52.85, 46.85, 29.71, 29.58, 28.32, 26.02, 21.25, 14.29. Purity: 98.23% by HPLC. HRMS (ESI): calcd for $(M+H)^+$ ($C_{25}H_{28}O_2N_2Cl^+$) 423.1834, found 423.1849.

ADMET studies of IR-780, MC-1 and MC-2

ADMET studies were conducted to predict the hepatotoxicity, absorbance and BBB penetration of three compounds. For ADMET studies, structures of all the compounds were built and full minimized using CHARMM force field using Discovery Studio 2.5 software (Accelrys, Inc., San Diego, Calif.). The low-energy conformers of all the compounds were used to calculate the ADMET properties. A plot was created using AlogP98 and 2D polar surface area (PSA_2D) of the compounds. The upper limit of PSA_2D in 95 and 99% confidence ellipses are 131.6 $Å^2$ and 148.1 $Å^2$. The upper limit of AlogP98 was 5.88 as defined by 95% confidence ellipse. The parameters of ADMET were judged according to the levels shown for each parameter. Blood brain barrier permeability (BBB) were classified by 0 for very high, 1 for high, 2 for medium, 3 for low and 4 for undefined. Intestinal absorption levels (HIA) were 0, 1, 2, and 3 which showed good, moderate, low, or very low absorption, respectively. ADMET aqueous solubility levels were six as 0 for extremely low, 1 for no; very low, but possible; 2 for yes, low; 3 for yes, good; 4 for yes, optimal, 5 for no, too soluble and 6 for molecules with one or more unknown AlogP98 types. Cytochrome P450 (CYP2D6) inhibition, hepatotoxicity were shown as 1 for the inhibitor and toxic or 0 for non-inhibitor and non-toxic, respectively. Plasma protein binding (PPB) was 0, 1, 2 which showed binding is < 90 %, > 90%, or > 95 %.

Extended discussion about ADMET studies: the absorption level of probes significantly

increased compared with IR-780. On the other hand, the hepatotoxicity of **MC-1** and **MC-2** was both 0, while IR-780 was 1, which suggested that our probes were less toxic than IR-780. More importantly, MC-1 and MC-2 were predicted to be inside 99% absorption confidence ellipsoid as well as the 99% BBB confidence ellipsoid (Fig. S9). These results proved that through replacing the cationic moiety with electrically neutral moiety, the probes showed higher BBB penetration, better absorption and lower hepatotoxicity.

General procedure of MTT assay

The MTT assay was used to measure the cytotoxicity of **MC-1** and **MC-2** to SH-SY5Y cells. Cells were seeded into a 96-well cell-culture plate. Then, the cells in each well were treated with various concentrations of probes, respectively, and were incubated at 37 °C under 5% CO_2 for 48 h. 10 μL MTT (5 mg mL^{-1}) was added to each well, and the cells were further incubated at 37 °C under 5% CO_2 for another 4 h. The MTT solutions were remove, and yellow precipitates (formazan) observed in plates were dissolved in 100 μL DMSO. Microplate reader was used to measure the absorbance at 570 nm for each well.

In Vitro Aβ Aggregates Binding Constant Determination

A solution of 5 μL of $A\beta_{1-42}$ aggregates (200 μM) was added to 245 μL of PBS solution containing **MC-1** (10-3000 nM in the final assay mixture). The mixture was incubated at room temperature for 1 h. The mixture was incubated for 10 min at room temperature, and the fluorescent intensity was measured (excitation: 630 nm; emission: 690 nm) by multifunction microplate reader. The value for the dissociation constant (K_d) was determined using GraphPad Prism 5.0 with nonlinear one-site binding regression (GraphPad Software, Inc., U.S.A.).

In Vitro Fluorescent Staining of Brain Slices

Paraffin-embedded 8 μm brain tissue sections from double Tg mice (C57BL6, APPswe/PSEN1, 22 month old, male) and age-matched wild-type mice (C57BL6, 22 month old, male) were used for in vitro fluorescent staining. Before staining, the slices

were deparaffinized by washing with ethanol for 5 min after a 15 min immersion in xylene. The slices were incubated in aqueous solution of MC-1 (1 μM) for 10 min at room temperature and then washed with ethanol/water (v/v=40%:60%). Next, the brain sections were observed using an Axio Observer Z1 (Zeiss, Germany) equipped with DAPI, AF488, AF546, and Cy 5.0 filter sets. To confirm the substantial staining of the plaques, adjacent section of Tg mice were stained with ThS (0.125% aqueous solution)

In Vitro Histological Staining of Brain Slices of Tg Mice. Paraffin-embedded 6-μm brain tissue sections from double Tg mice (C57BL6, APPswe/PSEN1, 22 months old, male) and WT mice (C57BL6, 22 months old, male) were used for in vitro fluorescence staining. Before use, the slices were well deparaffinized by washing with ethanol for 5 min after a 15 min immersion in xylene. The slices were incubated in solution of **MC-1** (1 μM, 2.5% DMSO, 10% ethanol) for 10 min at room temperature and then washed with ethanol/water (v/v, 40:60%). Next, the brain sections were covered with cover glasses prior to fluorescence observation using an Axio Observer Z1 (Zeiss, Germany) equipped with Cy 5.0 filter sets. To confirm the substantial staining of the plaques, adjacent sections were stained with Th-S (0.125% aqueous solution) using a GFP filter set.

In Vivo Imaging in Tg and WT Mice

In vivo imaging was performed using IVIS Lumina XR (Caliper Life Sciences). WT mice (C57BL6, 14-month-old, female) and double transgenic mice (C57BL6, APP/PS1, 14-month-old, female), used for *in vivo* imaging experiments, were purchased from Beijing HFK bioscience CO., LTD, Beijing, China. All animal study procedures were approved by the Sun Yat-sen University Animal Ethical Experimentation Committee according to the requirements of the National Act on the use of experimental animals (China).

Ex Vivo Histology Staining of Brain Slices

A 20-month-old Tg mouse (C57BL6, APPsw/PSEN1, male) and an age-matched WT mouse (C57BL6, male) were sacrificed 30 min after iv injection with **MC-1** (1

mg/kg, 20% DMSO, 80% propylene glycol, 100 μL), and the brain samples were excised, embedded in optimum cutting temperature compound (OCT), and frozen in dry ice immediately. For fluorescence observations, the brain samples were sliced into frozen sections of 20 μm. Fluorescent observation was performed by Axio Oberver Z1 (Zeiss) equipped with a Cy5 filter set. In addition, the Aβ plaques were further confirmed by the staining of the same section with ThS (0.125%) using a GFP filter set.

Computational Methods

Docking procedures were performed using the Schrodinger software (Schrodinger, LLC, New York, NY, USA) on widely used $A\beta_{(1-40)}$ fibril structures (PDB ID 2LMO). The optimized geometries of **MC-1** served as the input ligands, and the rigid protein ($A\beta_{1-40}$ fibril) was used as the macromolecule receptor. **MC-1** was drawn in Maestro (Schrodinger, LLC, New York, NY, USA), and optimized using the LigPrep package from Schrodinger, LLC, New York, NY, USA by means of the Optimized Potentials for Liquid Simulations (OPLS 2005) force field using the default setting, then subjected to Glide XP docking. Several studies have indicated that residues 16-KLVFFA-21 of the Aβ fibril could be the most preferential binding site for the interactions of small molecules with the fibrils. The length of enclosing box centered on this site was 36 Å. The most reliable pose of ligand was generated from 20 docking poses.

Determination of the Partition Coefficient

log P was determined by shaking-flask method. Octanol-water system was used to simulate lipo-hydro system. Octanol was saturated with water overnight. Then a series of concentration of **MC-1** solution were prepared using the octanol saturated with water. The absorbance of different concentration was measured. Working curve (absorbance *versus* **MC-1** concentration in octanol) was done in a linear range of 0.0025-0.03 mg/ml. 1 mg/ml **MC-1** in octanol was shaking with water for 12 h in dark and organic phase was diluted 40 folds to measure absorbance. The measurement was conducted in triplicate. Thus, the **MC-1** concentration in organic phase was determined. log P was given as the following equation:

$$\log P = \log \frac{[C_{organic\ phase}]}{[C_{water\ phase}]}$$

Photostability of MC-1

A solution of **MC-1** (10 mM in DMSO) was exposed under 254 nm UV light, lamplight, and sunlight respectively. Every 2 hours, 4 μL solution was diluted 1000-fold with methanol and fluorescent intensity was measured. The measurement was conducted in triplicate.

^{1}H NMR, ^{13}C NMR, MS, HRMS and HPLC spectra of compounds.

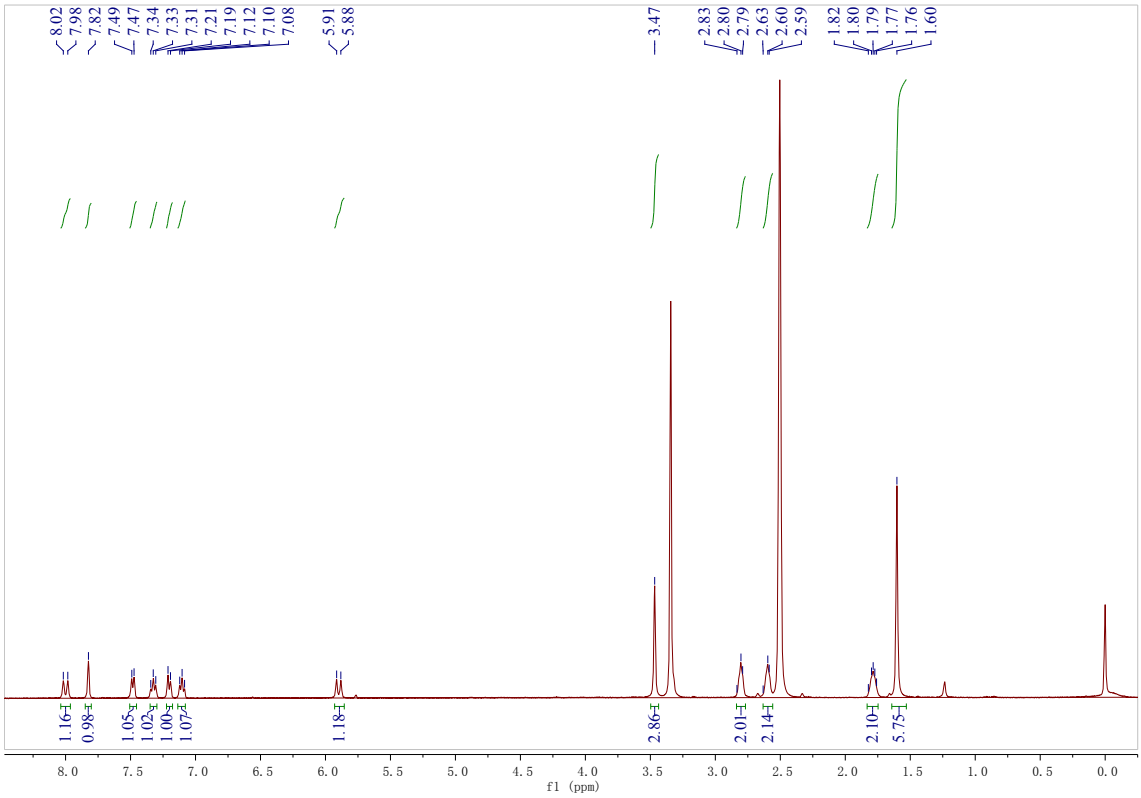


Fig. S1 ^{1}H NMR spectrum of **MC-1**

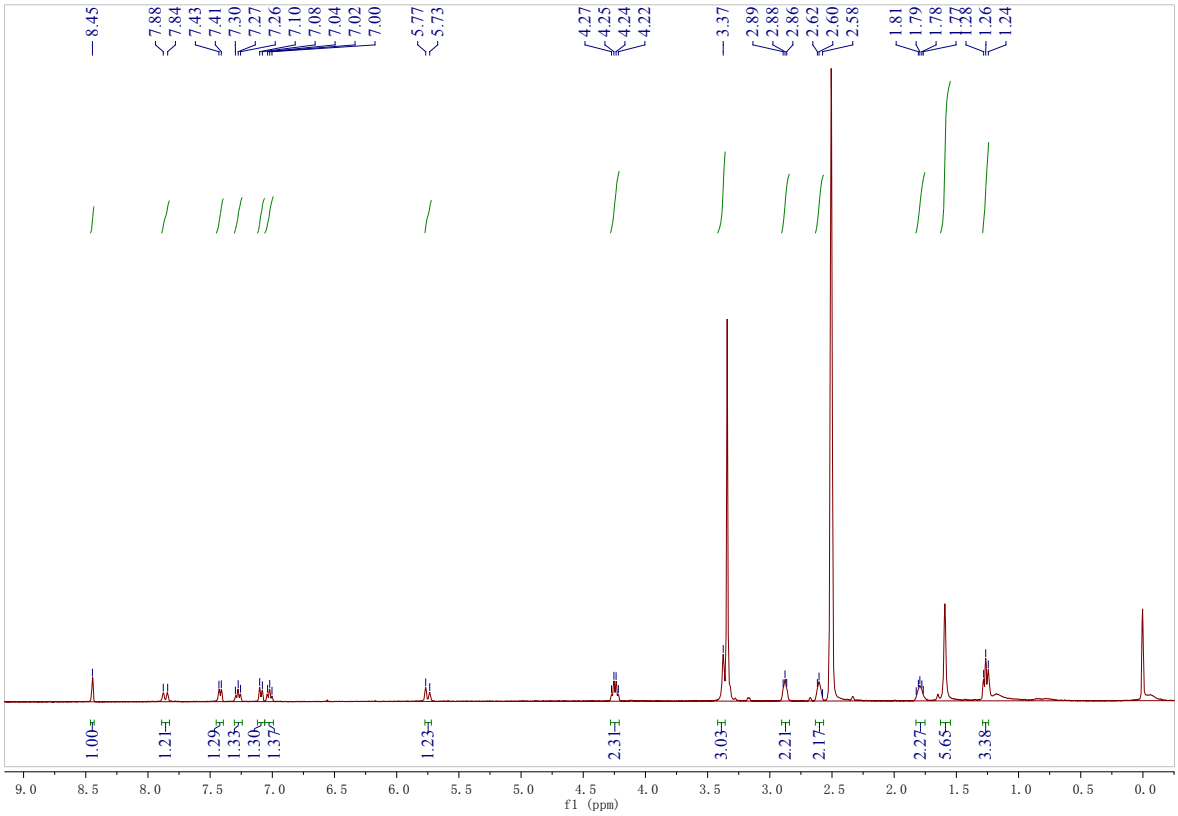


Fig. S2 ^{1}H NMR spectrum of **MC-2**

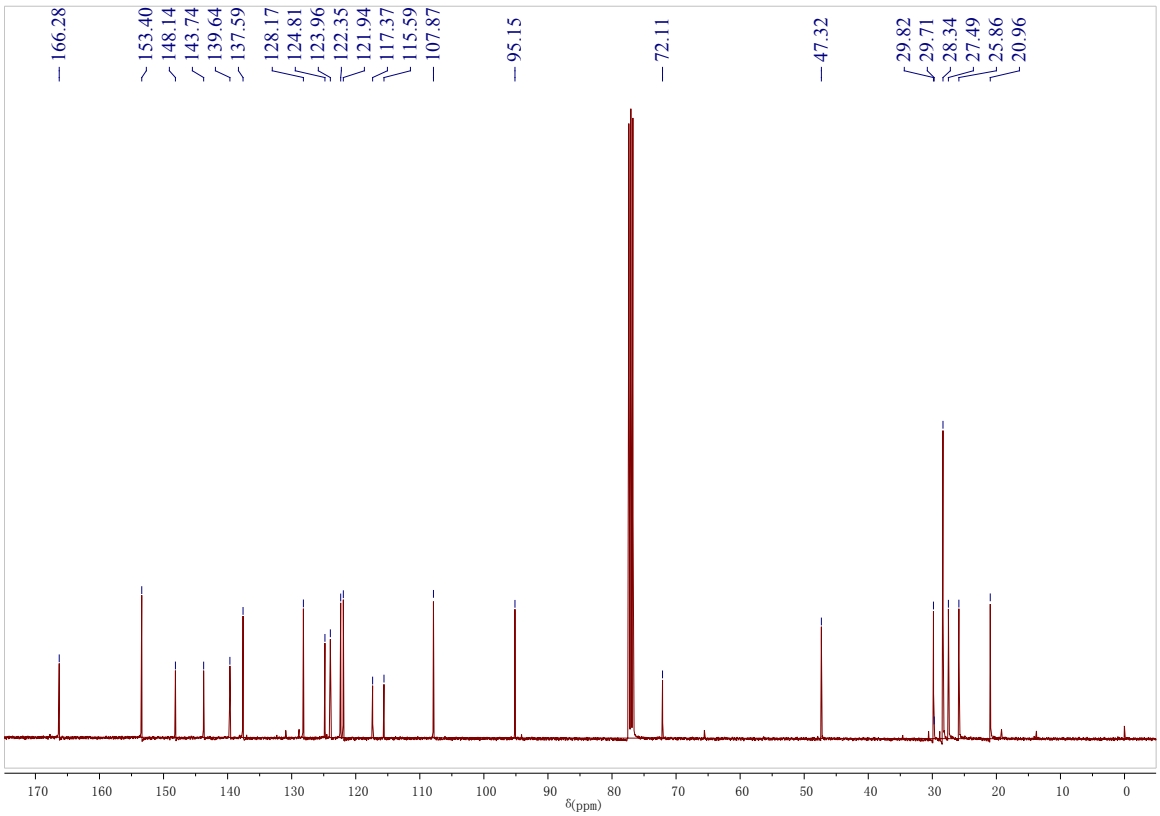


Fig. S3 ^{13}C NMR spectrum of **MC-1**

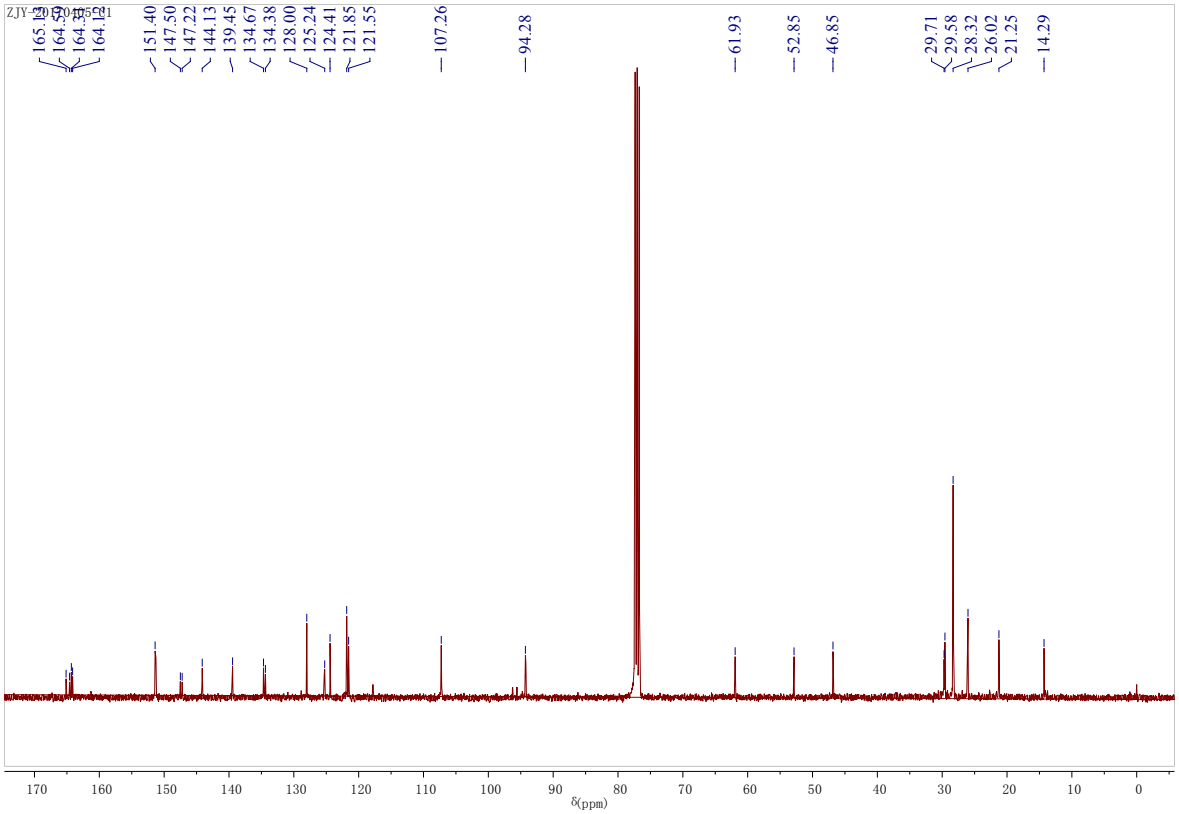


Fig. S4 ^{13}C NMR spectrum of **MC-2**

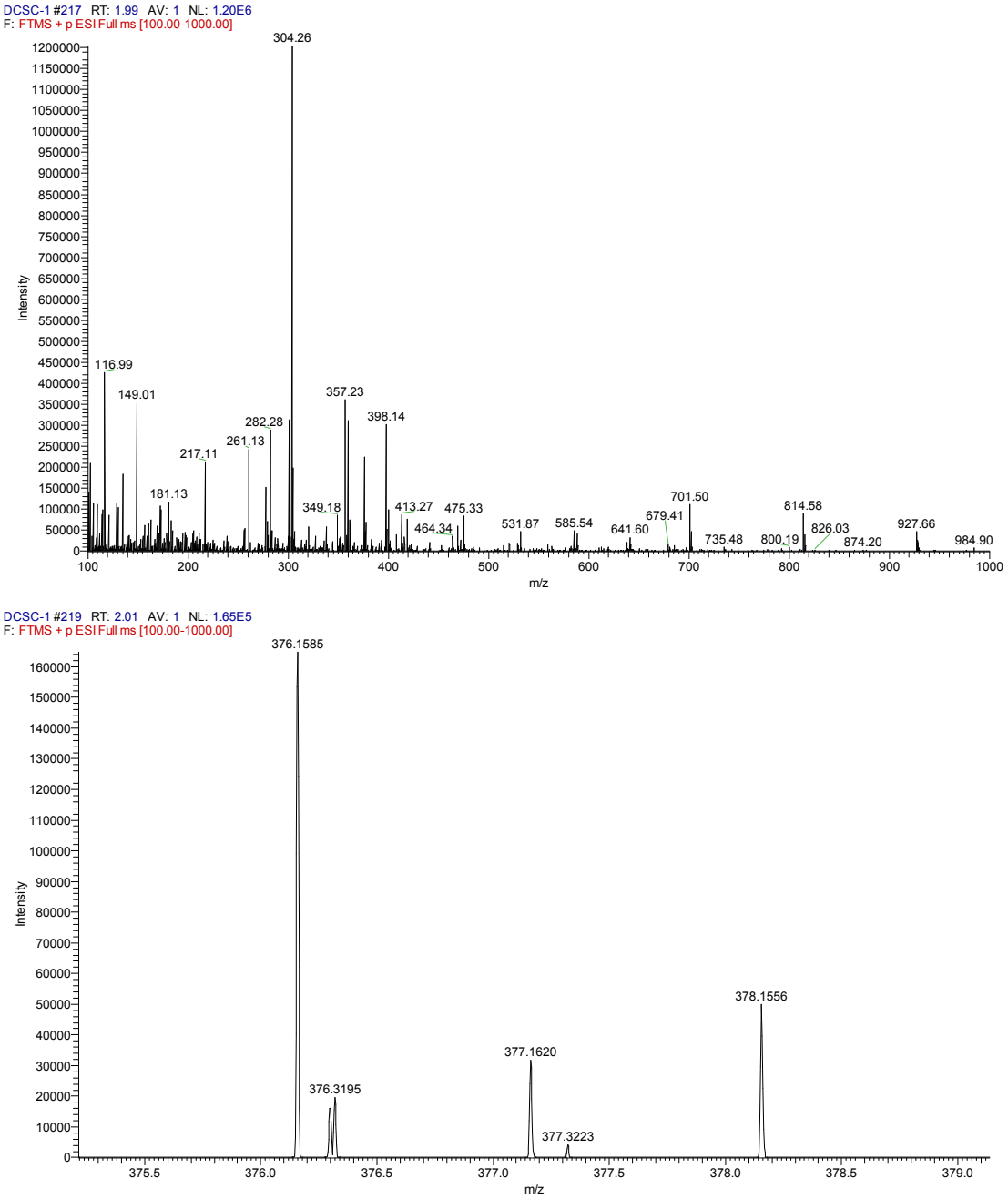


Fig. S5 HRMS spectrum of **MC-1**

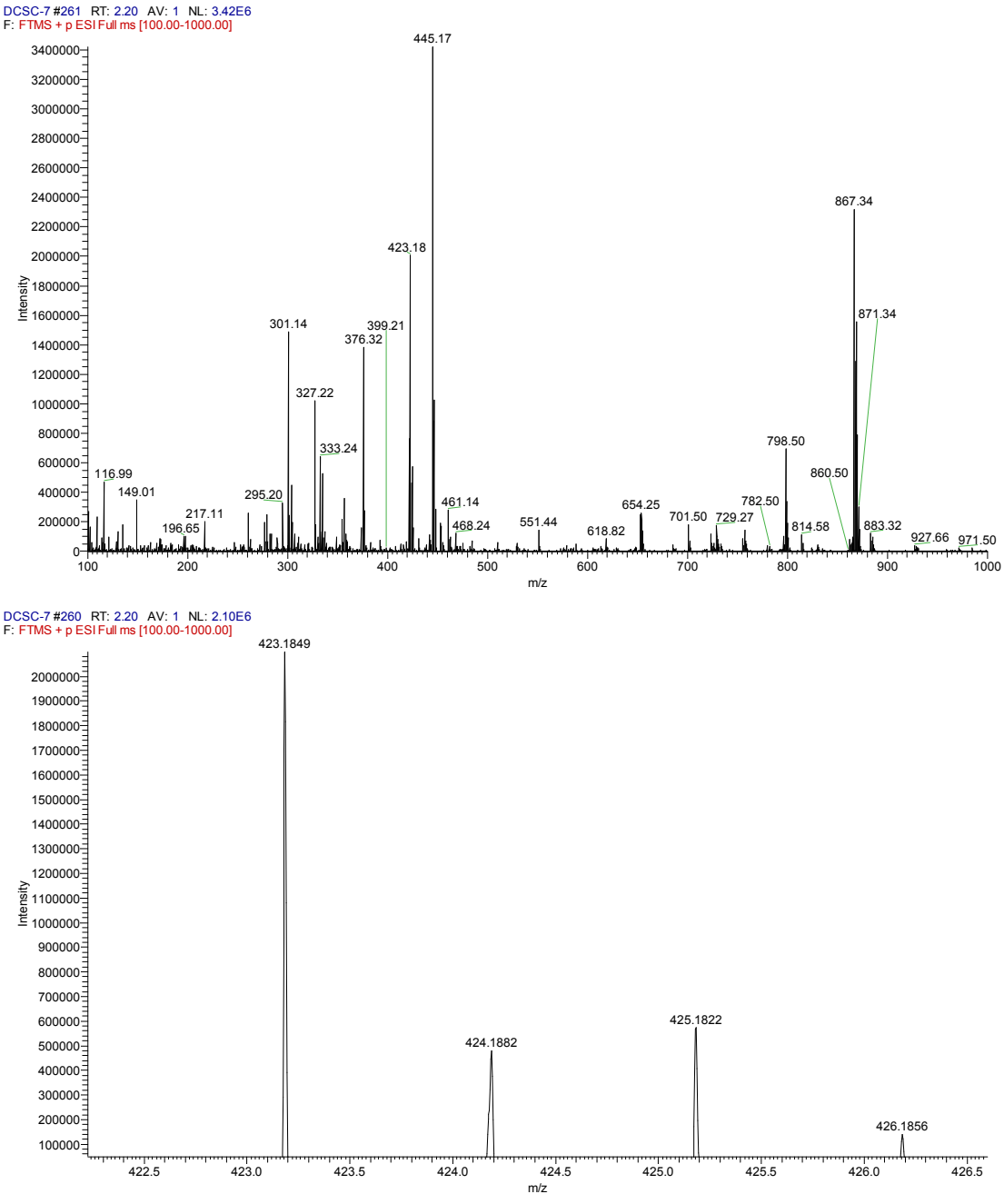
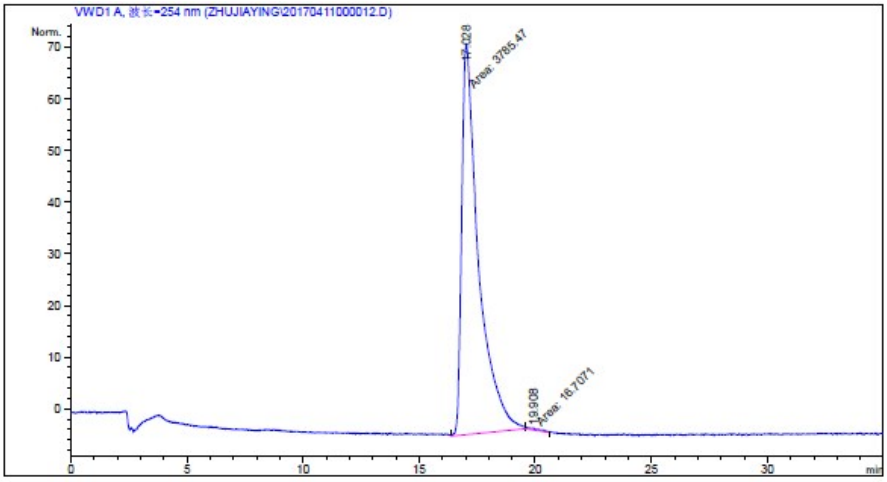


Fig. S6 HRMS spectrum of **MC-2**

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Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, 波长=254 nm

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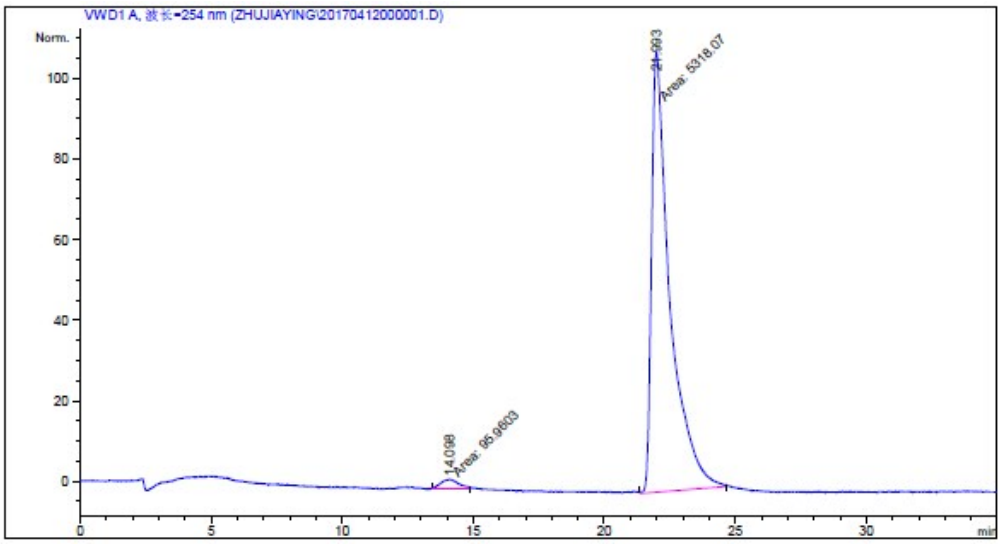
Fig. S7 HPLC spectrum of **MC-1**

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Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, 波长=254 nm

Peak RetTime Type  Width     Area      Height     Area
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Totals :                  5414.02619  111.46976

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Fig. S8 HPLC spectrum of **MC-2**

Table S1. ADMET properties of **MC-1**, **MC-2** and **IR-780**

Compounds	BBB	Absorption	Solubility	Hepatotoxicity	CYP2D6	PPB	AlogP98	PSA_2D
MC-1	0	0	1	0	0	2	5.2920	49.222
MC-2	0	0	1	0	0	2	5.6170	52.518
IR-780	4	3	0	1	0	2	7.8570	3.3520

Table S2. Fluorescent Properties, Binding Data, Calculated log P (clog P) Value and Measured log P of **MC-1** and **MC-2**

Probe	λ_{abs}[a](nm)	λ_{em1}[b](nm)	λ_{em2}[b](nm)	Φ (%)[c]	fold[d]	K_d (nM)[e]	clog *P*[f]	log P[g]
MC-1	630	695	685	1.50/0.42	28	59.09±5.6	4.79	3.70
MC-2	590	690	683	0.82/0.24	10	/	6.00	/

[a]Absorbance (λ_{abs}) measured in PBS. [b] Determined in PBS (λ_{em1}) and upon binding with Aβ aggregates (λ_{em2}). [c] Measured in DCM/PBS, respectively. [d] Fold increase in fluorescence intensity upon binding with Aβ aggregates. [e] K_d value was measured in triplicate with results given as the mean ± SD. [f] The values were calculated using the online ALOGPS 2.1 program. [g] The value was measured by conventional experiment.

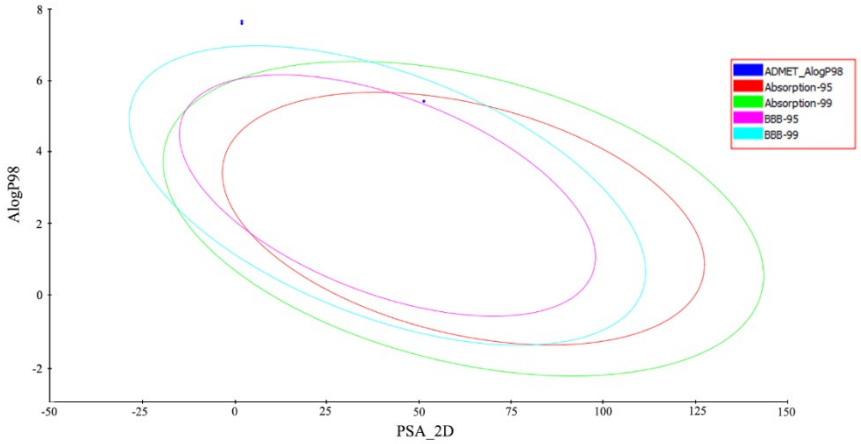

Fig. S9 Plot of PSA versus LogP with 95% and 99% confidence limit ellipses

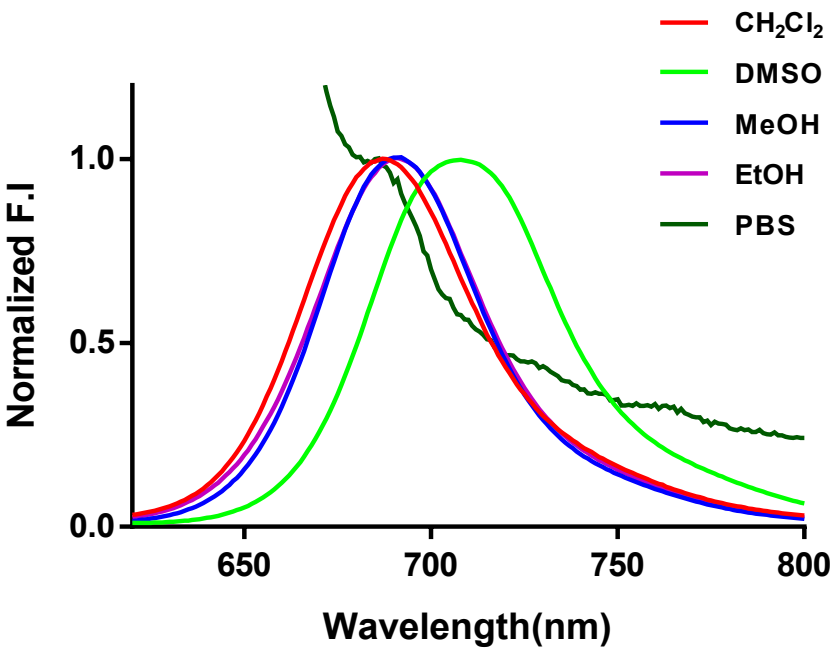

Fig. S10 Normalized fluorescence spectra of **MC-1** in different solvent.

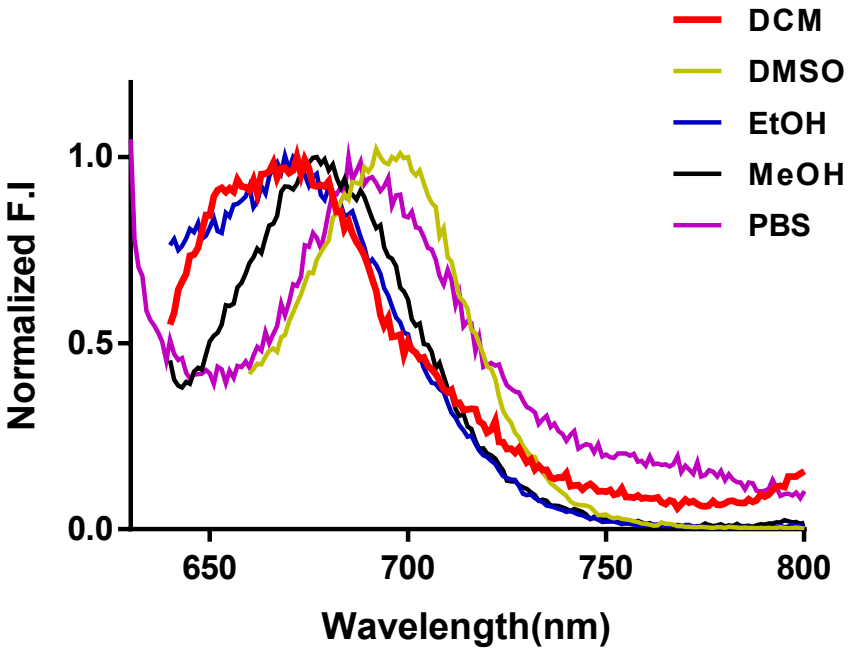

Fig. S11 Normalized fluorescence spectra of **MC-2** in different solvent.

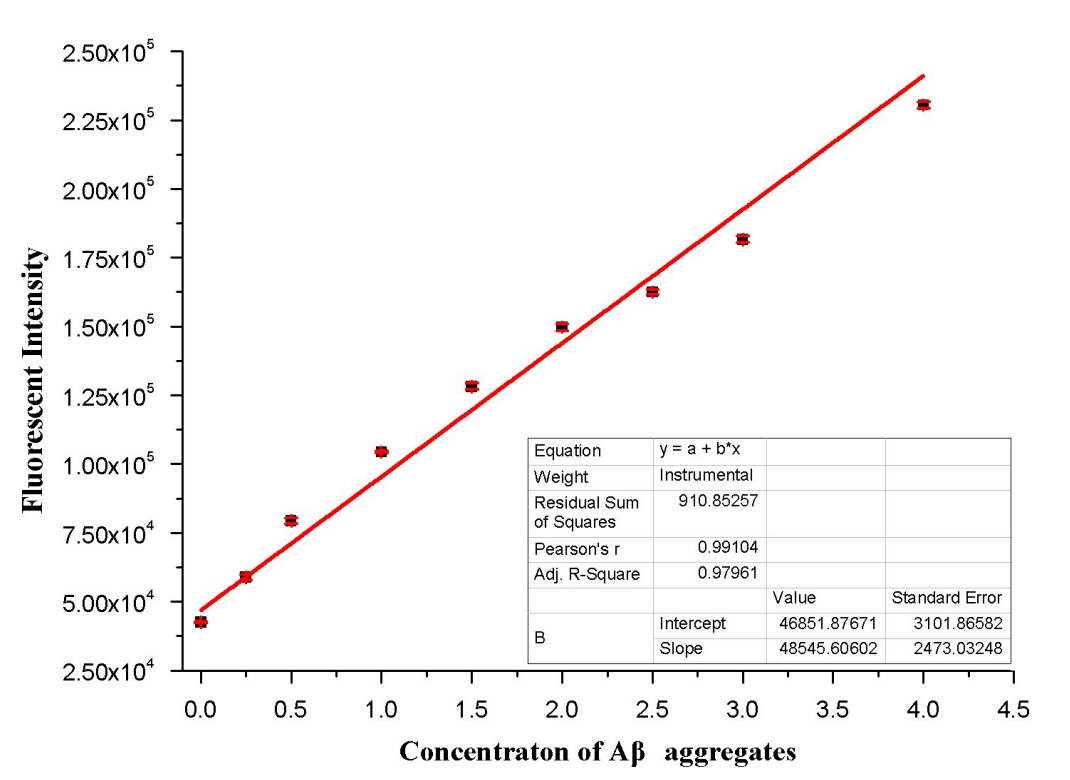

Fig. S12 Emission intensity at 685 nm of **MC-1** (1 μM) as a function of the concentration of Aβ aggregates in PBS buffer.

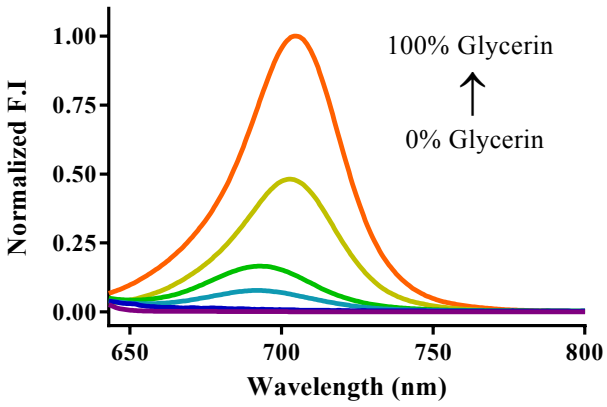

Fig. S13 Solvent viscosity-dependent fluorescence change of **MC-1** (5 μM in 0%-100% glycerin/PBS)

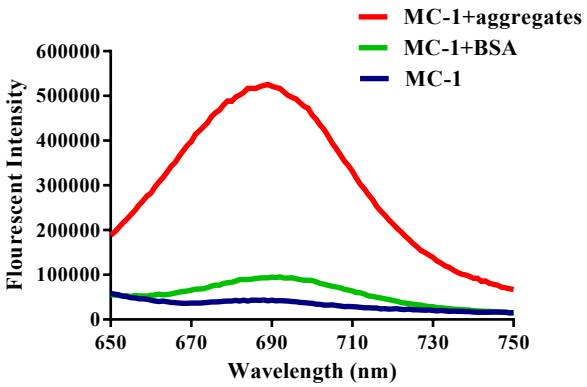

Fig. S14 Fluorescent Response of **MC-1** upon binding with Aβ aggregates and BSA in ACSF (Artificial cerebrospinal fluid).

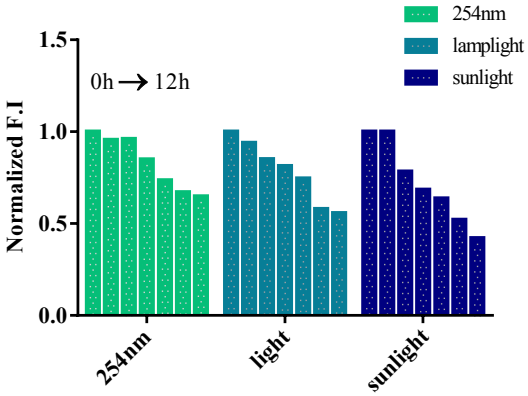

Fig. S15 Photostability of **MC-1** under UV (254 nm), lamplight and sunlight respectively (0, 2, 4, 6, 8, 10, 12 h).

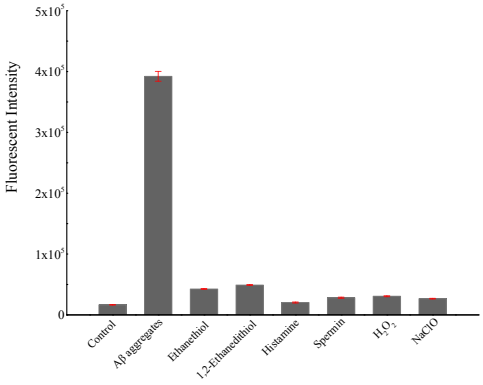

Fig. S16 The fluorescent spectral response of **MC-1** (1.0 μM) towards biological amines, thiols and typical ROS (5.0 μM) (in PBS, pH = 7.4).

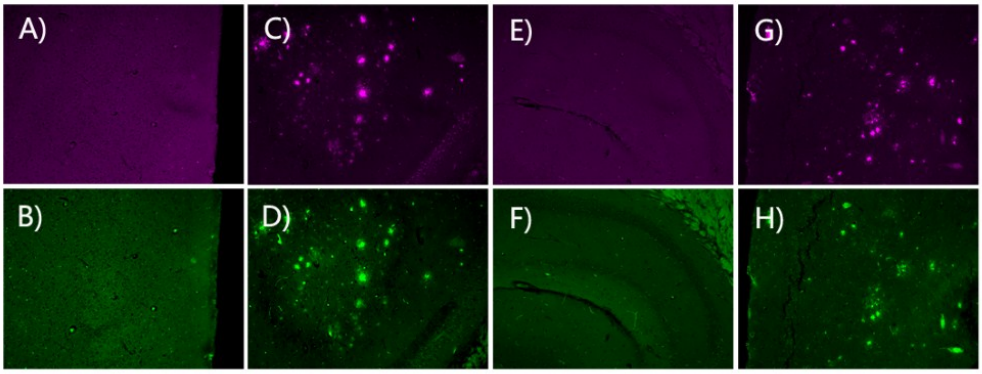

Fig. S17 In vitro fluorescent staining of brain slices of hippocampus region (A, C) and cortex region (E, G) from WT mice (A, E) and Tg mice (C, G) (C57BL6, APP/PS1, 22 months old, male). The Aβ plaques were confirmed by staining of adjacent sections with ThS (B, D, F, H). Magnification, 10×.

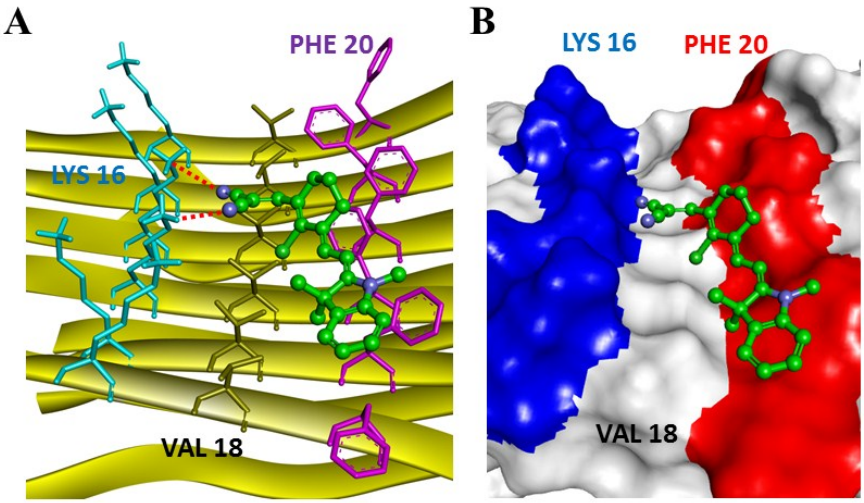

Fig. S18 Computational binding model of **MC-1** with 2-fold Aβ fibrils. The residues of Aβ fibrils are presented in the licorice style (A) and as a molecule surface representation (B).